



**Patent**

**IN THE UNITED STATES PATENT AND TRADEMARK OFFICE**

In re application of

**WEBBER, R.**

Examiner: HUFF, Sheela

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For: **IMMUNOASSAY METHOD EMPLOYING  
MONOCLONAL ANTIBODY REACTIVE  
TO HUMAN iNOS**

Atty Dkt: 14291

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**DECLARATION OF ROBERT WEBBER**

**(37 C.F.R. §1.132)**

I, **Robert Webber**, declare that I am the sole named inventor in the above-identified patent application. Prior to my invention therein, I attempted to obtain a monoclonal antibody which specifically binds to the native, whole human iNOS (inducible nitric oxide synthase) protein. The steps I followed began with the human iNOS amino acid sequence which was deduced from the DNA sequence. This involved ten different series of experiments spanning more than 2½ years. These experiments began in early 1993, at which time human iNOS protein was not available. Since iNOS differed from cNOS, later known as eNOS and nNOS, at the carboxyl and amino terminal regions, peptide analogues of the amino terminal region and

the carboxyl terminal region of human iNOS were designed, synthesized, purified and conjugated onto carrier proteins for use as immunogens. These experiments closely follow the suggestions noted in the cited Harlow and Lane reference.

The peptide/protein conjugates were injected into groups of both mice and rats in attempts to elicit an antibody response at the whole animal level that could in turn be used to develop an anti-peptide monoclonal antibody that would bind to the whole, native iNOS protein. Only after an antibody response in the whole animal was elicited to the peptide/protein conjugate was an animal used as a splenocyte donor in a fusion procedure with a suitable myeloma cell line in an attempt to develop a monoclonal antibody producing hybridoma. After selecting the hybridomas using the HAT selection technique, culture supernatant from each well of the master culture plates was tested to determine if it contained antibody that would bind to the peptide immunogen. Culture supernatant from each well that was found to be positive for anti-peptide antibody binding was then tested for antibody binding to the whole, native iNOS protein. The data for this series of experiments are summarized in Table 1, attached hereto as Exhibit A.

With respect to Exhibit A, each column of Table 1 is numbered at the top from (1) to (11) and each row except the top heading row is labeled (A) to (L) for ease of reference. The

information contained in each column is briefly described as follows:

Column 1 lists the start date for experiments performed on each group of animals, which is the date each group of animals received their first immunization with a peptide/protein conjugate.

Column 2 is the notebook number and page number corresponding to each of these groups of animals.

Column 3 lists the animal number series assigned to individual animals of each group of animals.

Column 4 is the species of the animals used in that experiment (either rats, mice or rabbits).

Column 5 is the region (carboxyl or amino) of the whole hiNOS protein of which the peptide immunogen is an analogue.

Column 6 lists the amino acid sequences used.

Column 7 lists the protein carrier used in the peptide/protein conjugate.

Column 8 is the antibody response found to the immunogen peptide when testing the whole polyclonal antiserum obtained from each animal of the group.

Column 9 lists the dates and notebook and page numbers for each hybridoma fusion procedure that was performed.

Column 10 lists the results of screening the culture supernatant from each master hybridoma well against the peptide immunogen.

Column 11 lists the results of testing the culture supernatant by ELISA from each of the positive wells identified by screening all master wells against the peptide (column 10) when such culture supernatant was tested for antibody binding to the whole, native hiNOS protein.

The supporting data for each row (A) through (L) are attached as Exhibits B-M, respectively.

Row A summarizes the data obtained from two separate hybridoma fusions using Rats # 2891 - 2894. The first fusion resulted in no positive hybrids being produced. The second fusion yield two potentially positive hybridomas (1E12 and 2E8) since they bound to the peptide immunogen, but no binding to the whole, native human iNOS protein was found. Exhibit B, data.

Row B summarizes the data obtained from a hybridoma fusion using Mouse # 2895. This fusion yield four potentially positive hybridomas (2B6, 2F3, 4F4 and 5E9) since culture supernatant was found to contain mouse antibody that bound to the peptide immunogen. However, no binding to the whole, native human iNOS protein was detected. Exhibit C, data.

Row C summarizes data from Mice # 2965 - 2967 in which no antibody response was elicited by the peptide/thyroglobulin conjugated immunogen. Exhibit D, data.

Row D summarizes data from Mice # 2968 - 2970 in which no antibody response was elicited by the peptide/thyroglobulin conjugated immunogen. Exhibit E, data.

Row E summarizes the data obtained from two separate hybridoma fusions using Rats # 3047 - 3050. The first fusion yield four potentially positive hybridomas (2C8, 2E8, 5F12 and 5H4) since culture supernatant was found to contain rat antibody that bound to the peptide immunogen. Exhibit F, data. However, no binding to the whole human iNOS protein was detected. The second fusion yield nine potentially positive hybridomas (1B11, 2C6, 3A6, 3F2, 3H8, 5G7, 6A8, 8A9 and 8B6) since they bound to the peptide immunogen, but no binding to recombinant human iNOS was detected. Exhibit F, data.

Row F summarizes data from Mice # 3271 - 3274 in which no antibody response was elicited by the peptide/thyroglobulin conjugate. Exhibit G, data.

Row G summarizes the data obtained from Mice # 3335 - 3340. This fusion yield 13 potentially positive hybridomas (1E5, 7E12, 8A4, 9H5, 11E1, 11F3, 12D3, 13B11, 16C12, 17A1, 17D7, 19A7, 19A8) since culture supernatant was found to contain mouse antibody that bound to the peptide immunogen, and two wells, 11F3 and 17D7, contained both mouse IgG and IgM antibodies. However, no binding to

the whole human iNOS protein was detected with any of these hybridomas. Exhibit H, data.

Row H summarizes data from Mice # 3962 - 3863 in which no antibody response was elicited by the peptide/thyroglobulin conjugated immunogen. Exhibit I, data.

Row I summarizes data from Mice # 4229 - 4231 in which no antibody response was elicited by the peptide/KLH conjugated immunogen. Exhibit J, data.

Row J summarizes data from Mice # 4232 - 4235 in which no antibody response was elicited by the peptide/KLH conjugated immunogen. Exhibit K, data.

Row K summarizes the data from Rabbits # 4098 - 4100 in which a strong antibody response was elicited using the same batch of peptide/KLH conjugate that did not elicit an antibody response in Mice #4232 - 4235. Exhibit L, data.

Row L summarizes the data from Rabbits # 4101 - 4103 in which a very strong antibody response was elicited using the same batch of peptide/KLH conjugate that did not elicit an antibody response in Mice #4229 - 4231. Exhibit M, data.

Table 1 of Exhibit A shows that not all of the mice and rats responded to the peptide/protein conjugates since no antibody was elicited in many of the animals even though these same peptide/protein immunogens were successfully used to elicit high titer antibody responses in groups of rabbits. This highlights two

points. First, since the rabbits described in rows (K) and (L) did respond with high titer antibody, this shows that the peptide/KLH immunogen was a good conjugate. Second, since all the rabbits responded and the mice did not respond, this emphasizes the species difference that exists and illustrates that what is a good immunogen in one species may fail completely in a different species. Since no antibody response was detected in these animals, they were not used as splenocyte donors for a hybridoma fusion procedure.

Of the six separate splenocyte-myeloma cell fusions performed, only five (Rows A, B, E, & G) yielded any hybridomas that produced antibody which bound specifically to the immunogen peptide: a total of 32 ( $2+4+4+9+13 = 32$ ) master wells were identified that contained either rat IgG, rat IgM, mouse IgG, or mouse IgM anti-peptide antibody. However, when culture supernatant from these wells of the master culture plates were tested for antibody that bound specifically to the whole native human iNOS protein, no antibody binding was detected.

In summary, over a period of more than 30 months, 29 mice and 8 rats were immunized with four different peptide analogues of the amino and carboxyl terminals of human iNOS as peptide/protein conjugates, in attempts to generate an anti-peptide monoclonal antibody that would bind to the whole, native human iNOS protein. No anti-peptide antibody that was produced by a hybridoma was ever detected as binding to native human iNOS, even though a total of 32

master wells (out of a total of 4800 master hybridoma wells) were determined to be producing antibody that could bind to the corresponding peptide immunogen.

I further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or documents or any registration issuing therefrom.

Date:

August 5, 2006

Robert Webber  
Robert Webber